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TECHNICAL NOTE

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Higher Failures of Amelogenin Sex Test in an Indian Population Group

ABSTRACT: The human sex test in forensic multiplexes is based on the amelogenin gene on both the X and Y chromosomes commonly used in sex genotyping. In this study of 338 male individuals in a Malaysian population comprising Malays, Chinese and Indians, using the AmpFℓSTR® Profiler Plus™ kit, the amelogenin test gave a significant proportion of null alleles in the Indian ethnic group (3.6% frequency) and 0.88% frequency in the Malay ethnic group due to a deletion of the gene on the Y chromosome. This sex test also failed in a forensic casework sample. Failure of the amelogenin test highlights the need for more reliable sex determination than is offered by the amelogenin locus in the Malay and Indian populations. The gender of the Indian-Malay amelogenin nulls was confirmed by the presence of three Y-STR alleles (DYS438, DYS390 and DYS439). For the Indian ethnic group, one of the Y-STR forms a stable haplotype with the amelogenin null. The amelogenin-deletion individuals also showed a null with a male-specific minisatellite MSY1, indicating that a very large deletion was involved that included the amelogenin and the MSY1 loci on the short arm of the Y chromosomes (Y_p).

KEYWORDS: forensic science, amelogenin, Y-STRs, MSY1, null allele, deletion

Simultaneous identification and sexing of human DNA samples using PCR profiling systems currently constitutes a major part of forensic DNA work. The sex test provided in commercial PCR kits, such as the AmpFℓSTR® Profiler Plus™ (PE Applied Biosystems, Foster City, CA) is based on the amelogenin gene, which is located on the X chromosome ($X_p22.1–22.3$) and the Y chromosome ($Y_p11.2$) (1). The X-Y homologous region of the amelogenin gene codes for a protein involved in the formation of the tooth enamel matrix (2,3). In PCR systems that utilize the amelogenin gene, sex determination is based on the detection of the two versions of this gene, which generally differ by a 6 bp deletion between the X and Y chromosomes (4). This PCR-based sex test is popular, as it is sensitive, easy to interpret, and can be co-amplified with other loci in a single multiplex.

In the AmpFℓSTR® Profiler Plus™ system, the presence of both the X (106 bp) and Y (112 bp) copies in almost equal proportions indicates a male genotype while the presence of only the X copy indicates a female genotype. Absence of the X copy does not give incorrect sexing results, as the Y copy still indicates the presence of a Y chromosome and therefore a male genotype (5). However, absence of the Y copy will cause incorrect interpretation of gender based on this sex test alone, particularly if the source of the DNA is unknown as in forensic evidential samples.

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Our study of 338 male individuals from three major ethnic groups in the Malaysian population showed a significant drop out of the amelogenin Y copy in a particular ethnic group and none in another group. This paper presents the frequency of this null allele, the confirmation of sex using Y-linked STRs, and the affinity of a particular Y-STR/amelogenin Y null haplotype. The null alleles were challenged using different amelogenin primer sequences and primers for an adjacent locus, the Y-specific minisatellite MSY1, which is located on the same p-arm as the amelogenin locus. Since the challenges also failed, the lesion involving the amelogenin Y was much larger than a point mutation. Failure of the amelogenin sex test, particularly in an evidential sample from a crime scene, showed that other distant loci on the Y chromosome should be considered in the sexing of human DNA samples besides the amelogenin marker. Incorrect sex genotyping due to this null allele in forensic evidences may hamper or discredit the investigative process if other information is not taken into account.

Materials and Methods

DNA Source and Preparation

Blood samples stored on FTA® paper from 338 unrelated male individuals (comprising 113 Malays, 113 Chinese and 112 Indians), and a forensic evidence sample, were provided by the Malaysian Chemistry Department. One FTA® disk (1.1 mm in diameter) was excised out of each paper. Genomic DNA was directly amplified from these samples using a standard processing protocol for FTA® paper (two washes with 200 μL of 10 mM NaOH, followed by two washes with 200 μL of 0.1X TE, and then dried at 56°C for 20 min). DNA from the evidential sample was extracted using the Chelex method (6).

AmpFℓSTR® Profiler Plus™ Analysis

The DNA samples were amplified with the AmpFℓSTR® Profiler Plus™ (PE Applied Biosystems, Foster City, CA) according to the manufacturer's instructions in a GeneAmp® PCR System 9600 (Applied Biosystems). One washed FTA® disk (1.1 mm in diameter) was used in the PCR. For the forensic evidence sample, quantitation was not performed and 5 μL (from 80 μL) of Chelex extract was used. The PCR products were analyzed on the ABI PRISM™ 377 (PE Applied Biosystems, Foster City, CA).

Analysis of Amelogenin Null Samples

1. Amplification of AMEL-2F/3R—The amelogenin null samples and the evidence sample were amplified with a combined amelogenin primer pair (AMEL-2F/3R) from Sigma Genosys, Australia:

AMEL 2F: 5′ ACCTCATCCTGGGCACCCTGG 3′

AMEL 3R: 5′ TAAAGAGATTCATTAACTTGACTG 3′

The AMEL-2F/3R primer sequences spanned the full region of the three common amelogenin primer-annealing sites shown in Fig. 1 of Roffey et al. 2000 (1). Table 1 gives a summary of the PCR mix and the cycling conditions. The amplified products were separated on a standard 1% agarose gel and stained with ethidium bromide. Designation of the respective X and Y alleles was determined by visual comparison with at least two known male and female controls. The presence of two bands at 1174 bp and 985 bp denotes a male profile, whereas female profiles only show a single band at 1174 bp.

2. Amplification of MSY1 Locus—The highly polymorphic Y-specific minisatellite MSY1 (DYF155S1) was also amplified in the above samples using a modified primer pair:

Y1A+: 5′ ACAGAGGTAGATGCTGAAGCGGTATAGC 3′

Y1E+: 5′ ATTAATTGAGGTTGTTGTGCATACAGAT 3′

The forward primer Y1A+ is from Jobling et al. (7) while the reverse primer Y1E+ is redesigned. Amplification was performed in 25 μL reaction volume. See Table 1 for the amplification mix and PCR conditions. The amplified MSY1 products were separated on a 1.2% agarose gel and stained with ethidium bromide. Male samples show the minisatellite MSY1 product between 1.5–2.5 kb while female samples do not show this product.

3. Amplification of Y-STRs—Three Y-STRs that are distant to the amelogenin gene (DYS438, DYS390 and DYS439) were optimized and amplified in the null samples. Figure 1 shows a schematic diagram of the locations of the Y-STRs (DYS438, DYS390 and DYS439) with respect to the amelogenin gene, the MSY1 locus, and other male-specific markers that have been used in the sex determination of human DNA samples. The DYS390 primer sequence was from the Y-STR Haplotype Reference Database (http://www.ystr.org), while the DYS438 and DYS439 primer sequences were from Ayub et al. (8). The forward primers of the three Y-STRs were labeled with fluorescent dyes (DYS438 with 6-FAM, DYS390 with JOE, and DYS439 with TAMRA) by Genset Oligos, Australia. Amplification was carried out in 15 μL reaction volumes. See Table 1 for the amplification mix and PCR conditions. Designation of Y-STR alleles was achieved by using two Y-STR control samples with known allele repeat numbers, kindly supplied by M. A. Jobling, University of Leicester, UK.

Results and Discussion

From the 338 male samples analyzed with the Profiler Plus™ multiplex, four Indian males and one Malay male showed a dropout of the 112 bp amelogenin Y allele (*AMEL Y*) (See Fig. 2*A*). The casework sample involved a murder victim who was severely burnt. Bloodstains found on the murder weapon were transferred to cotton gauze fibers. The Profiler Plus™ result from the bloodstain matched with that from the muscle specimen of the victim. This sample also showed a complete *AMEL Y* dropout. However, all six amelogenin null samples, including the casework sample, gave Y-STR alleles (Fig. 2*B*), verifying that the five males did in fact carry the Y-chromosome, and that the DNA source on the murder weapon had come from a male.

Amplification of the Indian and Malay samples with the combined AMEL-2F/3R primers (Fig. 2*C*) showed that the amelogenin null was not due to a point mutation at the primer-binding sites, but was caused by a deletion involving the amelogenin locus on the Y chromosome. The evidence sample did not produce any detectable amelogenin PCR fragments. Based on the fact that males should

TABLE 1—*Details of the amplification mixtures and PCR cycling conditions.*

System	Amplification Mixtures	PCR Cycling Conditions
AMEL 2F/3R	30 μL volume containing 0.50 μM AMEL 2F/3R primers, 1X GeneAmp® PCR Buffer II (PE Applied Biosystems, containing 10 mM Tris-HCl pH 8.3, 50 mM KCl), 1.5 mM $MgCl_2$, 200 μM of each dNTPs (PE GeneAmp®), 1.2 U AmpliTaq Gold™ polymerase (PE Applied Biosystems) and 16 mg/mL BSA (Sigma Chemical Co.)	1X 95°C, 11 min; 15X (94°C, 0.45 min; 70°C, 1.5 min); 25X (94°C, 0.45 min; 52°C, 1.5 min; 70°C, 1 min), and 1X 72°C, 10 min.
MSY1	30 μL volume containing 0.15 μM of Y1A+ and Y1E+ primers, 1X GeneAmp® PCR Buffer II (PE Applied Biosystems, containing 10 mM Tris-HCl pH 8.3, 50 mM KCl), 1.67 mM $MgCl_2$, 200 μM of each dNTPs (PE GeneAmp®), 1.2 U AmpliTaq Gold™ polymerase (PE Applied Biosystems) and 16 mg/mL BSA (Sigma Chemical Co.)	1X 95°C, 11 min, 28X (94°C, 0.5 min; 65°C, 1 min; 68°C, 3 min*), and 1X 68°C, 30 min. * :increase 10sc/cycle
Y-STR triplex	15 μL volume containing 0.13 μM DYS438 primers, 0.10 μM DYS390 primers, 0.50 μM DYS439 primers, 1X GeneAmp® PCR Buffer II (PE Applied Biosystems, containing 10 mM Tris-HCl pH 8.3, 50 mM KCl), 1.5 mM $MgCl_2$, 200 μM of each dNTPs (PE GeneAmp®), 1.2 U AmpliTaq Gold™ polymerase (PE Applied Biosystems) and 16 mg/mL BSA (Sigma Chemical Co.)	1X 95°C, 11 min; 30X (94°C, 1 min; 57°C, 3 min; 72°C, 3 min), and 1X 72°C, 20 min.

Note: Amplification of the Y-STRs was performed in a GeneAmp® PCR Systems 9600 (Applied Biosystems) while amplifications of the AMEL 2F/3R and the MSY1 locus were performed in a GeneAmp® PCR System 2400 (Applied Biosystems).

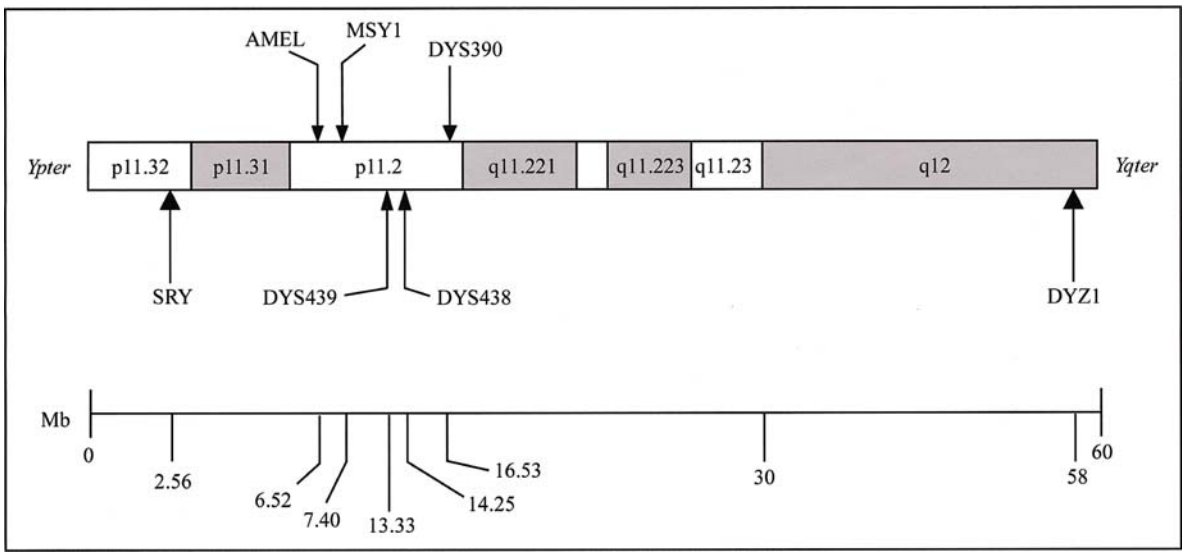

FIG. 1—*Schematic diagram showing the locations of the three Y-STRs with respect to the amelogenin gene, and the MSY1 locus, on the human Y chromosome. Other male-specific markers that are commonly used for sex determination of human DNA samples, such as the SRY and DYZ1 are also shown. From the left are the SRY (Sex-determining region Y) gene, the AMEL (Amelogenin) gene, the minisatellite MSY1 (DYF155S1) locus, the three Y-STR loci (DYS439, DYS438 and DYS390) and the DYZ1 locus. An approximate distance in megabases between the loci was obtained from the Ensembl Human Genome Browser and is indicated by the scale below the diagram.*

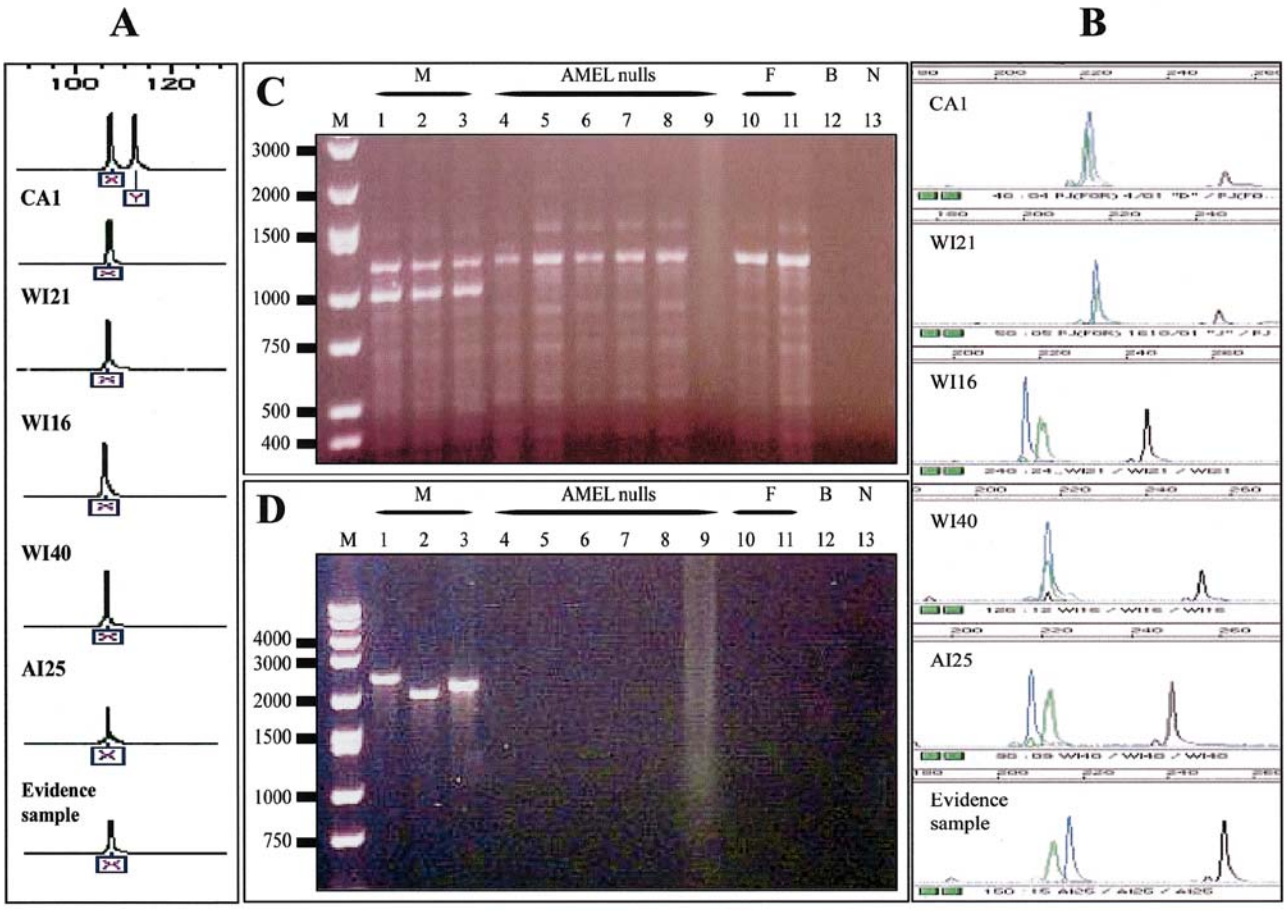

FIG. 2—(A) *AmpF*ℓ*STR® Profiler Plus™ result shows the dropout of the AMEL Y in the five male individuals (CA1, WI21, WI16, WI40, AI25) and the evidential bloodstain. The top panel is a male control showing the X and Y alleles at the amelogenin locus.* (B) *Y-STR analysis (in GeneScan™) of the six amelogenin-deletion samples. Note that for CA1, WI16, and the evidential bloodstain, there is a size overlap between the DYS438 and DYS390 alleles, but both can be determined from the different fluorescent dye tags.* (C) *PCR products with AMEL-2F/3R primers show a deletion of the 985 bp product in the five male individuals (CA1-lane 4, WI21-lane 5, WI16-lane 6, WI40-lane 7, AI25-lane 8). Note that the evidential bloodstain (lane 9) did not yield detectable PCR fragments but only shows a smear in the gel lane. The three male control samples (lanes 1–3) show both the 1174 bp X product and the 985 bp Y product, while the two female control samples (lanes 10 and 11) show only the 1174 bp X product. Other lanes contain: 12 = zero DNA (1 blank disk); 13 = negative control (no disk); M = Sigma DNA Marker (New England Biolabs).* (D) *PCR products of the MSY1 locus show a deletion of the 1.5–2.5 kb minisatellite in the five male individuals, and no conclusive result in the evidential bloodstain, which only shows a smear in the gel lane. Figure legends are the same as in* C.

have two alleles (X and Y) when amplified with the AMEL-2F/3R primers, and the X allele was not even observed on the gel, it can be concluded that the negative result for this sample is most likely due to the very low level of template that was available on that piece of fiber sample. In addition, all five amelogenin-deletion males also showed a null at the male-specific MSY1 minisatellite (Fig. 2*D*). The drop out of both the *AMEL Y* and the MSY1 loci in these individuals indicated that the deletion spanned a significant length on the p-arm of the Y chromosome, probably in the order of kilobases. A deletion of about 1 Mb in the short arm of five Y-chromosomes exhibiting *AMEL Y* null was also observed by Thangaraj et al. (9).

The failure rate of the amelogenin sex test in the studied Indian population group is 3.57%, which is higher than observed in the Malay (0.88%), and Chinese (0%) groups. The higher amelogenin deletion frequency in the Indian population in Malaysia appears to be real as it has independent and prior support from Santos et al. (10), and recently from Thangaraj et al. (9). A deletion frequency of 8% was observed by Santos et al. (10), where two out of 24 Indian male individuals of Sri Lankan origin exhibited the *AMEL Y* deletion. In Thangaraj et al. (9), five out of 270 Indian male individuals exhibited this similar phenomenon, indicating a deletion frequency of 1.85%. A recent study in a Caucasian population from Austria showed five males with *AMEL Y* deletion from a total of 28,182, indicating a much lower deletion frequency of only 0.018% (11). Although the size of the studied population datasets is very different, particularly in Santos et al., the common failures of this sex test in several global Indian groups is still worthy of note.

Interestingly, the four amelogenin-deletion Indian individuals in this study had the same DYS438*allele 9, as did the two Sri Lankan individuals from Santos et al. (personal communication from M. A. Jobling, 2002). This suggests the possibility that the DYS438*allele 9 and the *AMEL Y* deletion form a relatively old and stable haplotype in the Indian ethnic group. This is indicated because all six *AMEL Y* deletion individuals have the DYS438*allele 9, but the other two Y-STRs by contrast, gave a variety of haplotypes with the *AMEL Y* deletion (see Table 2). The frequency of DYS438*allele 9 in the studied Indian population was 39/112=0.35 (Table 3).

It would be useful to know if the amelogenin Y null shows population-specific difference. Several other studies have shown this phenomenon existing in two Sri Lankan Indians in Santos et al. (10), an Australian Caucasian in Roffey et al. (1), a Moroccan father-son pair in Henke et al. (12), six Austrians in Stinlechner et al. (11), and five Indians from India in Thangaraj et al. (9). In addition, a Spaniard, a Slovenian and two individuals from the UK, also exhibited the *AMEL Y* null (personal communication from M. A. Jobling, 2002). These four European individuals did not have the same *AMEL Y* null/DYS438* allele 9 haplotype as the six Indian individuals, indicating independent origins as well as reinforcing the observed stable haplotype above in the individuals from Indian origin.

A rare converse amelogenin null has also been reported, where the 106 bp amelogenin X copy (*AMEL X*) is absent in three out of 7220 (0.04%) male samples (5). By using the AMEL-2F/2R primers, the X-alleles were amplified in these three null samples, indicating that the *AMEL X* null is due to a point mutation at the primer-binding site rather than a deletion on the X chromosome.

Other alternative methods for sex determination in human DNA samples include the use of the *SRY* or sex-determining region Y gene on the Y_p (9–11), the *DYZ1* satellite III on the Y_q (1,2), and X-Y STR loci (13,14). The *SRY* gene, although on the same Y p-arm as the amelogenin, is highly conserved in humans (15) unlike the amelogenin gene, which is functionally diploid and has no known male-specific functions. X- and Y-STR systems with polymorphic copies on both the X and Y chromosomes, for example, the multi-allelic DXYS156, also have use in sex identification and allow geographic inferences to be made (13).

The use of male-specific Y-STRs with fluorescent dye technology provides a rapid and reliable confirmatory test for the presence of the Y chromosome, as it is compatible with current forensic technology that uses sensitive automated platforms. The ability to amplify Y-STRs in all six *AMEL Y* deletion samples, including the limited amount of template from the evidential bloodstain, could be due to the smaller amplicon sizes and the higher sensitivity of the Y-STR triplex, which can reach 0.1 ng (16). This indicates that the Y-STR system is suitable to analyze trace DNA or low copy number forensic samples that fail the amelogenin sex test.

In conclusion, failure of the amelogenin test indicates the need for the inclusion of more reliable Y-loci in sex determination, as gender misidentification, at least in some populations, is going to have an unacceptable frequency if based on the amelogenin test alone. Although amelogenin failures are well reported, failure of this sex test at a frequency of 3.6% in the Malaysian Indians is high enough to be more than just noteworthy. As an alternative, the three Y-STRs appeared to be useful for gender verification, and the peculiar haplotype of *AMEL Y* deletion/DYS438*allele 9 could pro-

TABLE 3—*Allele frequencies of DYS438 in the Indian ethnic group.*

DYS438 Allele	Number	Frequency
8	1	0.009
9	39	0.348
10	40	0.357
11	30	0.268
12	2	0.018
13	0	0
14	0	0
Total	112	1

Note: The observed allele frequencies above in the Indian population group were extracted from Chang et al. 2002 (17).

TABLE 2—*Y-STR haplotypes of the amelogenin-deletion individuals.*

Sample	Amelogenin	DYS438 Allele	DYS390 Allele	DYS439 Allele	Ethnic Origin of Sample
CA1	X -	10	25	13	Malay
WI21	X -	9	25	11	Indian
WI16	X -	9	24	13	Indian
WI40	X -	9	25	12	Indian
AI25	X -	9	23	13	Indian
Evidence bloodstain	X -	9	24	11	Indian*

* The ethnic origin of the evidence bloodstain is based on the matching Profiler Plus™ result of the stain with the victim, who was of Indian origin.

vide extra-assurance of a true null in the Indians. For forensic casework, the Y-STR confirmation of male phenotype will be of relevance in prenatal sex tests, paternity cases, and in criminal investigations where the victim has identified the assailant as an Indian male but the crime scene samples obtained appear to show only a female DNA source.

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